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Patterns of gene expression in pig adipose tissue: Insulin-like growth factor system proteins, neuropeptide Y (NPY), NPY receptors, neurotrophic factors and other secreted factors☆

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Abstract

Although cDNA microarray studies have examined gene expression in human and rodent adipose tissue, only one microarray study of adipose tissue from growing pigs has been reported. Total RNA was collected at slaughter from outer subcutaneous adipose tissue (OSQ) and middle subcutaneous adipose tissue (MSQ) from gilts at 90, 150, and 210 d ($n = 5$ age^{-1}). Dye labeled cDNA probes were hybridized to custom porcine microarrays (70-mer oligonucleotides). Gene expression of insulin-like growth factor binding proteins (IGFBPs), hormones, growth factors, neuropeptide Y (NPY) receptors (NPYRs) and other receptors in OSQ and MSQ changed little with age in growing pigs. Distinct patterns of relative gene expression were evident within NPYR and IGFBP family members in adipose tissue from growing pigs. Relative gene expression levels of NPY2R, NPY4R and angiopoietin 2 (ANG-2) distinguished OSQ and MSQ depots in growing pigs. We demonstrated, for the first time, the expression of IGFBP-7, IGFBP-5, NPY1R, NPY2R, NPY, connective tissue growth factor (CTGF), brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF) genes in pig adipose tissue with microarray and RT-PCR assays. Furthermore, adipose tissue CTGF gene expression was upregulated while NPY and NPY2R gene expression were significantly down regulated by age. These studies demonstrate that expression of neuropeptides and neurotrophic factors in pig adipose tissue may be involved in regulation of leptin secretion. Many other regulatory factors were not influenced by age in growing pigs but may be influenced by location or depot.

Keywords: Adipose tissue; Porcine; Gene microarray; Secreted factors; Receptors

1. Introduction

Adipose tissue secretes a wide variety of proteins that include adiponectin, leptin, insulin-like growth factors (IGFs) (reviews, 1–4) and insulin-like growth factor binding proteins (IGFBPs; 5–7). Additional factors expressed or secreted by adipose tissue include interleukins and other cytokines such as vascular endothelial growth factor (VEGF) and angiopoietins (reviews, 4,8,9). Through the secretion of these factors, adipose

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tissue is involved in a number of physiological and metabolic processes (reviews, 1,2,4). Expression of these factors and related genes by adipose tissue has been studied with cDNA microarrays in human and rodent studies [9,10]. Since many of these secreted factors, such as the IGFBPs, can have opposing or synergistic actions [11], it is critical to examine as many as possible concurrently in the same study. A cDNA microarray reverse transcription (RT)-PCR approach was used to examine gene expression of four groups or families of secreted factors, i.e., transforming growth factor beta super-family members, interferons, interleukins and apolipoproteins in adipose tissue from the pigs used in the present study [12]. In this report, gene expression of an additional 18 secreted factors in adipose tissue from growing pigs was examined with gene microarray and RT-PCR analysis.

With an approach that included gene microarray and proteomic technologies, many secreted proteins in 90-d fetal stromal-vascular cultures and subcutaneous adipose tissue from 105-d fetuses and 5-d-old pigs were identified [13]. Several groups of these secreted factors, i.e., transforming growth factor beta super-family members, interferons, interleukins and apolipoproteins were followed up in studies of adipose tissue from these pigs [12]. In neonatal studies [13] numerous receptors including receptors for leptin, growth hormone (GH), IGFs and neuropeptide Y were also identified (NPY; Hausman GJ, unpublished observations). In the present study 8 receptors identified in neonatal adipose tissue studies in addition to 18 secreted proteins (13; Hausman GJ, unpublished observations) were followed up with gene expression studies of these secreted factors and receptors in adipose tissue from growing pigs. Furthermore, outer subcutaneous adipose tissue (OSQ) and middle subcutaneous adipose tissue (MSQ) depots were compared in regards to relative gene expression patterns for these receptors and secreted factors given that cellular and metabolic development distinguishes these depots [14] as does gene expression for a number of cytokines and apolipoproteins [12]. The studies presented herein represent initial efforts to identify adipose tissue secreted factors that may regulate puberty and growth in pigs.

2. Material and methods

2.1. Animals

Data in the present and a previous study [12] were generated from the same tissues and pigs so details of animal handling and care are reported elsewhere [12]. Briefly, five PIC (Pig Improvement Company, Hendersonville, TN) composite lean phenotype gilts were utilized at 90, 150, and 210 d, for a total of 15 pigs [12]. The expected time of puberty was approximately 210 d which was consistent with age related changes in expression of pituitary and hypothalamic genes. Mean BW (kg) were 35 ± 5 at 90 d, 82 ± 5 at 150 d, and 127 ± 5 at 210 d which were significantly different from each other. Gilts were housed under conventional conditions and fed according to recommended guidelines prior to slaughter [12]. All procedures were approved by the Richard B. Russell Agriculture Research Center Committee on Animal Care and Use.

2.2. Gene expression analysis

Details of the following procedures are published [12] so the following materials and methods descriptions are abbreviated for the sake of brevity.

2.3. Tissue collection and RNA preparation

Five pigs at 90, 150, and 210 d of age were euthanized (a total of 15 pigs) with an overdose of 10% sodium thiopental and OSQ and MSQ were collected at slaughter. Total RNA was isolated from frozen tissues and RNA quantity and quality was determined. A dye swap design was used to correct for dye-specific bias during hybridization. Transcribed cDNA replicates were labeled with either Cy3 or Cy5 dye and the dye swap replicates were hybridized to different array slides.

2.4. Pig adipose tissue microarrays

Custom cDNA microarrays (Telechem International Inc., ArrayIt.com, Sunnyvale, CA) were prepared by spotting 70-mer oligonucleotides that were designed from 560 pigs gene sequences as described [12,13]. Slides (microarrays) were prehybridized and then hybridized to the labeled targets using a 25% formamide solution at 42 °C overnight. Microarrays were scanned and signal output was reported with image analysis software. Microarray intensities were adjusted for local background intensities which were low and comparable for OSQ and MSQ microarrays [12]. Normalization among arrays was performed with a version of a global mean normalization procedure as described [13].

2.5. Relative expression of normalized microarray gene intensities

Normalized microarray intensities were averaged, resulting in 5 average intensities (i.e., 5 pigs) per gene per tissue at each age. A gene within the IGFBP and NPYR gene group was arbitrarily designated as a reference gene for that gene group and set at 100. Percentages of the average intensity of the reference gene were calculated for the other genes in the group resulting in 5% per gene at each age. Subsequently, we compared relative gene expressions (percentages) to each other within the IGFBP and NPYR group at each age for MSQ and OSQ depots. Percentages from 90, 150 and 210 d microarray data were analyzed for age effects for MSQ and OSQ. Since an age effect was not observed we combined gene percentages from 90, 150 and 210 d pig adipose tissue microarrays and compared relative gene percentages to each other within the IGFBP and NPYR groups for OSQ and MSQ. Percentages were also computed with a second reference gene to determine if the choice of a reference gene influenced gene expression patterns. Our microarrays did not contain family or group members of the other genes studied so we normalized the expression of these genes with one of the following reference genes, i.e., IGF-I, fibroblast growth factor (FGF)-1 or lactate dehydrogenase (LDH)C depending on the gene. We used either IGF-I or FGF-1 as a reference gene for relative expression of genes encoding secreted factors whereas LDHC was used as a reference gene for all other genes. The expression of IGF-I, FGF-1 and LDHC in microarrays was similar and constant across ages so the reference gene used had no influence. For instance, using LDHC as a reference gene for secreted factor genes, instead of IGF-I, had no influence on the results of the comparisons made. Collectively, percentages of a total of 120 genes in 20 gene groups were computed and analyzed from 90, 150 and 210 d OSQ and MSQ microarray data.

Table 1
Identities of genes examined in outer and middle subcutaneous adipose tissue microarrays from 90, 150 and 210 d old pigs

Gene	Acc# TIGR[a]	Identification
Secreted factors		
	TC185594	Insulin-like growth factor I precursor (IGF-I)
	TC183976	Insulin-like growth factor II precursor (IGF-II)
	TC191294	Fibroblast growth factor 7 (FGF-7)
	TC174734	Acidic fibroblast growth factor (a FGF)
	NP276134 Singleton	Insulin-like growth factor binding protein 5 (IGFBP-5)
	NP275375 Singleton	Insulin-like growth factor binding protein 3 (IGFBP-3)
	NP275499 Singleton	Insulin-like growth factor binding protein 2 (IGFBP-2)
	TC184281	Insulin-like growth factor binding protein 6 (IGFBP-6)
	TC15832	Insulin-like growth factor binding protein 7 (IGFBP-7)
	TC20146	Angiopoietin 2 (ANGPT2)
	TC10402	Vascular endothelial growth factor A precursor (VEGF-A)
	TC282152	Connective tissue growth factor precursor
	TC21820	Putative preproadipsin
	TC162733	Follistatin precursor (FST)
	TC164670	Relaxin (relaxin)
	TC186429	Brain-derived neurotrophic factor (BDNF)
	TC163286	Small inducible cytokine A2 precursor (CCL2)
Receptors		
	TC11221	IGF-1 receptor (Sus scrofa)
	NP275996 Singleton	Mannose-6-phosphate/insulin-like growth factor II receptor (Fragment)
	TC16330	Neuropeptide Y1 receptor (NPY1R)
	TC12111	Neuropeptide Y2 receptor (NPY2R)
	TC44483	Neuropeptide Y receptor type 4 (NPY4-R)
	TC10439	Neuropeptide Y receptor type 5 (NPY5-R)
	TC8839	Leptin receptor precursor (LEP-R)(OB-R)
	TC15207	Growth hormone receptor precursor (GHR)
Others		
	BFO80718	Neurofilament light polypeptide (NEFL)
	TC10435	Stearoyl-CoA desaturase (SCD)

[a] TIGR, The Institute of Genome Research, TC and NP accession numbers can be searched at: http://www.tigr.org/tigr-scripts/tgi/T_reports.cgi?species=pig.

2.6. Determination of expression intensity ratios between the 90 and 150 d, 150 and 210 d and 90 and 210 d age groups

Microarrays were scanned and signal output reported as described above. Microarray intensities from each of 5 pigs/age group were utilized with 2 data points/pig (Cy3, Cy5 dye swapping replicates) totaling 10 microarray intensities/age. Background corrected fluorescent intensities were normalized by a global mean normalization and averaged for each gene and age. These averages were used to compute expression ratios between the ages for each gene. The accession numbers and identity of all the genes examined in these studies are shown in Table 1.

2.7. Quantitative real-time reverse transcription-PCR

Real-time reverse transcription (RT)-PCR was performed by the University of Georgia Functional Genomics Resource Facility and carried out using the Applied Biosystems 7900HT Sequence Detection System. Primers and probes were custom-designed, based on GenBank sequences of target genes, by Applied Biosystems (Foster City, CA; Table 2). Porcine 18S rRNA was amplified as an endogenous control. Five hundred nanograms of total RNA was transcribed into complementary deoxyribonucleic acid (cDNA) and was diluted 1:20 for use in the amplification reaction. The amplification reaction consisted of 1 μl diluted cDNA, 6.25 μl 2× TaqMan® Universal PCR Master Mix, 0.625 μl 20× custom TaqMan® Gene Expression Assay, and water up to a total volume of 12.5 μl. Thermal cycling parameters included 40 cycles of denaturing-annealing/elongating.

C_t (threshold cycle) values were determined using Applied Biosystems 7900HT Version 2.2.1 Sequence Detection Systems software, in relative quantification study mode, with thresholds set manually. C_t values (within-sample triplicate wells averaged) were then exported and subjected to two analyses:

$2^{-\Delta\Delta C_t}$ *method*. C_t values for replicates of each age group were averaged. ΔC_t was calculated as the difference in C_t between the target gene and the 18S endogenous control. $\Delta\Delta C_t$ was calculated as the difference in ΔC_t between the sample of interest and a calibrator sample; in this case, the sample of interest was the older age and the calibrator sample was the younger age. The differential expression ratio of the older to younger age groups for each gene was calculated as $2^{-\Delta\Delta C_t}$. Ratios are reported as $2^{-\Delta\Delta C_t}$ if upregulated or -1 divided by $2^{-\Delta\Delta C_t}$ if downregulated to correct for nonlinear appearance, with positive numbers indicating upregulation and negative numbers indicating downregulation [15].

REST© *method*. For calculating probe efficiencies within the REST© software tool, pooled cDNA of all available pig samples were run in quantitative real-time PCR reactions using 1/20, 1/40, 1/60, and 1/80 dilutions of TaqMan® probes. C_t values for the experimental samples, along with C_t values for the probe efficiencies, were imported into the REST© software tool. Replicates of each age group were not averaged. The REST© software tool determined differential gene expression ratios of the older to younger age groups for each gene, which were corrected for varying probe efficiencies as calculated by the software. REST© estimated p-values using the software's statistical model, a Pair-Wise Fixed Reallocation Randomisation Test©. The software reports ratios as $2^{-\Delta\Delta C_t}$ if upregulated or -1 divided by $2^{-\Delta\Delta C_t}$ if downregulated. The equation used within the software:

$$\text{ratio} = \frac{(E_{\text{target}})^{\Delta C_t \text{ target(mean control}-\text{mean sample)}}}{(E_{\text{ref}})^{\Delta C_t \text{ ref(mean control}-\text{mean sample)}}}$$

where E = probe real-time PCR efficiency, target = target gene, ref = endogenous control gene, in this case 18S. Mean CPs (crossing points; equivalent to C_t's) from the REST© analysis are shown to demonstrate gene expression [16].

2.8. Real-time RT-PCR reactions performed on middle and outer subcutaneous adipose tissue microarray mRNA

cDNA templates were designed to study a total of 13 genes present on our gene microarrays and were chosen based on expression in microarrays of neonatal adipose tissue and fetal stromal-vascular cell cultures (13; Hausman et al., 2007, unpublished observations). These genes were: VEGF, ANG-2, connective tissue growth factor (CTGF), IGFBP-5, IGF-II, IGF-I, IGFBP-7, brain-derived neurotrophic factor (BDNF), FST, NPY1R, NPY2R, LEPR, and GHR (Table 1). cDNA templates were also designed to study two other genes, i.e., ciliary neurotrophic factor (CNTF) and NPY (Table 1). We identified CNTF protein in conditioned media [13] and others demonstrated immunoreactive NPY protein [17,18] and NPY mRNA (dot blot analysis; 19) in adipose tissue. Reverse transcription-PCR reactions were performed on 90 and 210 d MSQ mRNA for all of these genes and on 150 d MSQ mRNA for several of these genes. RT-PCR reactions were performed on 90 and 210 d OSQ mRNA for several of these genes.

Table 2
Primer pairs for each selected gene, reporter 1 sequence and Genebank accession number from which the primer pairs were selected

Gene	Symbol	GenBank accession #	1. Forward primer sequence (5′ to 3′), 2. Reverse primer sequence (5′ to 3′), 3. Reporter 1 sequence (5′ to 3′)	Design strand
Follistatin	FST	NM_001003662	1. GGAAGTCCAGTACCAAGGCAAAT, 2. GCTGCCTGGACAGAAAACATC, 3. CCGACAGGTCTTTTTAC	Reverse
Growth hormone receptor	GHR	NM_214254	1. GGACTGTGGATCAAAAGTGTTTCTC, 2. GTTGAGGCCAATGGGTGGAT, 3. CCGGTTGCACTATTTC	Reverse
Vascular endothelial growth factor	VEGF	NM_214084	1. TGAGCTTCCTACAGCACAACAAAT, 2. GGGATTTTCTTGCCTCGCTCTATC, 3. TTTCTTTGGTCTGCATTCAC	Reverse
Insulin-like growth factor binding protein-5	IGFBP-5	NM_214099	1. GTTTGCCTCAACGAAAAGAGCTA, 2. GTGCTCGCGGGAGTCT, 3. TCTCGATCTTGGCTTGCTC	Reverse
Connective tissue growth factor	CTGF	NM_213833	1. GTGTGACGAGCCCAAGGA, 2. GGGCCAAACGTGTCTTCCA, 3. CCTGGCGGCTTACCG	Forward
Insulin-like growth factor binding protein-7	IGFBP-7	TC162689	1. CGTCACCCAGGTCAGCAA, 2. GCACCAGTGACATTCCAGATG, 3. CACGATGGAAGGACCTTG	Reverse
Ciliary neurotrophic factor	CNTF	U57644	1. CGTTCAGACCTGACTGCTCTTAT, 2.GTCCAGGTTGATGTTCTCATTCAGA, 3. ATGCTTCACATAAGCTTCC	Reverse
Insulin-like growth factor- I	IGF-I	NM-214256	1. GCGCCACACGGACATG, 2. TCCTGAACTCCCTCTACTTGTGTT, 3. CAAGGCTCAGAAGGAAGTA	Forward
Insulin-like growth factor- II	IGF-II	NM-213883	1. GGACACCCTCCAGTTTGTCT, 2. GCTGCGGCGGTTCAC, 3. CCGGCCTGCTGAAGTA	Reverse
Neuropeptide Y	NPY	AF264083	1. TCGGCGTTGAGACATTACATCA, 2. GTCTCGGGACTAGATCGTTTTCC, 3. CACCAGGCAGAGATAC	Forward
Neuropeptide Y Y1 receptor	NPY1R	NM_214288	1. CTGCAGTATTTTGGACCACTTTGTT, 2.TGTCTCTCATTTTGTCCATCATGTTGT, 3.TATGTAGATCTTAAAGTAGCAAATAA	Reverse
Neuropeptide Y Y2 receptor	NPY2R	NM_214150	1. GTGTGTGGTGGTGGTATTTGC, 2. TGTCAATGTCCACAGCAAGCT, 3. CCTCTGCATGCCTTCC	Forward
Brain-derived neurotrophic factor	BDNF	NM_214259	1. TGTATACGTCCCGAGTCATGCT, 2.GTATTCCTCCAGCAGAAAGAGAAGAG, 3. CTCCAAAGGCACTTGACTG	Reverse
Angiopoietin 2	ANG2	NM_213808	1.CAGACCAGTGAAATAAACAAATTGCAAGA, 2. AGTTGAACTATGTGCTTGTCTTCCAT, 3. CAGTTTCCTGGAAAAGAA	Forward
Leptin receptor	LEPR	NM_001024587	1. GATTCTCCACCAACATGTGTCATTC, 2. ATTTCTGCTTTCACACTGGATGGA, 3. TTTCACCACGGAATCAG	Reverse
Relaxin	RLN	NM_213872	1. AAGAGCCTCAGCTGGAAACTG, 2. AGATTTCTGCATCTTTGGTGATGGA, 3. CCGGCAGAAACCATG	Forward
18S ribosomal RNA	18S	NR_002170	1. AGGGCATCACAGACCTGTTATTG, 2. CCCCAACTTCTTAGAGGGACAAG, 3. CAGCCACCCGAGATTG	Reverse

2.8.1. Statistics

The relative expression levels of genes (percentages) within 2 groups or families of genes were analyzed with a two-way ANOVA for the effects of age, gene, and age × gene interactions using PROC GLM (SAS Inst. Inc., Cary, NC) or with a one-way ANOVA for comparing relative gene expressions (percentages) with each other within a gene group at each age using PROC GLM (SAS Inst. Inc.). Relative expression data from all three age groups were combined and analyzed with a one-way ANOVA for comparing relative gene expressions to each other within a gene group using PROC GLM (SAS). Differences between means were determined by the least squares contrasts using the PROC GLM procedure. Differences in gene expression determined by real-time RT-PCR were analyzed using a pairwise fixed allocation randomization test, utilizing the relative expression software tool (REST© version 2). All means were normalized to the endogenous control 18S rRNA expression. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Adipose tissue gene expression ratios between 90 and 150 d, 150 and 210 and 90 d and 210 d age groups

Expression ratios clearly indicate little change in gene expression with age in MSQ and OSQ since an expression ratio of 1 indicates no change (Table 3). Analysis of these microarray data with either a LOWESS normalization-based or an ANOVA normalization-based method showed that expression of these genes was not significantly ($P > 0.001$) influenced by age whereas CTGF was significantly increased ($P = 2.8 \times 10^{-5}$) with age in OSQ but not MSQ (Hausman et al., 2007, unpublished observations).

3.2. Patterns of gene expression in subcutaneous adipose tissue from 90, 150 and 210 d old pigs

We combined relative IGFBP and NPYR family gene expressions from 90, 150, 210 d pig adipose tissue microarrays for both OSQ and MSQ (Table 4). Comparing expression of genes to each other within the IGFBP and NPYR families and comparing MSQ and OSQ gene expressions indicated gene expression patterns (Table 4). The pattern of expression of IGFBP genes was depot-dependent and characterized by predominant IGFBP-5 expression (percentage) in the both depots coupled with an intermediate level of IGFBP-3 expression only in the MSQ depot regardless of the reference gene (Table 4). The NPYR expression pattern for growing pigs was also depot-dependent since NPYR percentages were different from each other to a greater degree in the MSQ than in the OSQ depot (Table 4). In particular, the relative expression of NPY2R and NPY4R were lower in OSQ than in MSQ (Table 4). Comparison of OSQ and MSQ depots showed that expression of several genes were also influenced by depot (Table 4).

The relative expression of two adipocyte markers, a secreted factor (preproadipsin) and a key enzyme stearoyl-CoA desaturase (SCD) was similar for OSQ and MSQ (Table 4). Surprisingly, adipose tissue expression of neurofilament light polypeptide (NEFL) was considerable and depot-dependent (Table 4).

3.3. Age-independent gene expression in middle subcutaneous adipose and outer subcutaneous adipose from growing pigs

Mean crossing points (CPs, equivalent to crossing thresholds) generated during REST© analysis were used to demonstrate expression of genes that were not influ-

Table 3
Average expression ratios of gene groups for three age comparisons of middle (MSQ) and outer (OSQ) subcutaneous adipose tissue microarray data[a]

Genes	Ages					
	MSQ			OSQ		
	90-150 d	150-210 d	90-210 d	90-150 d	150-210 d	90-210 d
IGFBPs (6)	1.07 ± 0.24	0.92 ± 0.19	0.96. ± 0.12	1.1 ± 0.15	1 ± 0.08	1.15 ± 0.11
IGF-I, -II, FGF-1, -7, FST, preproadipsin	0.98 ± 0.14	0.98 ± 0.21	0.98 ± 0.31	1.1 ± 0.17	1 ± 0.12	1.06 ± 0.18
VEGF, ANG-2, BDNF, SCYA2	1.05 ± 0.21	1.08 ± 0.28	1.08 ± 0.26	1.05 ± 0.16	1.08 ± 0.06	1.14 ± 0.22
NPYRs (4)	0.96 ± 0.04	0.88 ± 0.04	0.84 ± 0.004	1.1 ± 0.13	1 ± 0.11	1.12 ± 0.18
LEPR, GHR, IGF-1R, IGF-IIR	1.02 ± 0.10	0.99 ± 0.22	1.03 ± 0.35	1.16 ± 0.07	1 ± 0.07	1.2 ± 0.11
FGF1R, FGF2R, FGF4R	0.94 ± 0.11	1.01 ± 0.2	0.94 ± 0.13	0.97 ± 0.11	1.1 ± 0.16	1.03 ± 0.2

[a] Means ± S.D. of several IGFBPs, NPYRs, other receptors, hormones and growth factors. An expression ratio of 1 indicates no change with age. Expression ratios represent microarray data from 5 pigs/age group.

Table 4
The relative expression patterns of adipocyte markers, cytokines, NPY receptors, and IGFBPs for middle (MSQ) and outer (OSQ) adipose tissue based on combined microarray data from 90, 150 and 210-d-old-pig[a]

Gene	MSQ	OSQ	*P* value*
Preproadipsin	395 ± 35	465 ± 33	NS
SCD	161 ± 18	149 ± 21	NS
NEFL	1061 ± 120	549 ± 80	0.001
ANG-2	187 ± 20	125 ± 11	0.01
SCYA2 (CCL2)	234 ± 36	158 ± 15	0.06
NPY1R	100 b	100 b	
NPY2R	99 ± 3 b	87 ± 4 c	0.004
NPY4R	110 ± 3 c	88 ± 5 ce	0.002
NPY5R	121 ± 6 cd	104 ± 7 be	NS
IGFBP-1	100 b	100 b	
IGFBP-2	101 ± 4 b	98 ± 7 b	NS
IGFBP-3	139 ± 9 c	117 ± 8 b	NS
IGFBP-5	168 ± 19 d	203 ± 32 c	NS
IGFBP-6	121 ± 7 bc	114 ± 10 b	NS
IGFBP-2	100b	100b	
IGFBP-1	100 ± 3 b	109 ± 9 b	NS
IGFBP-3	139 ± 10 c	122 ± 6 b	NS
IGFBP-5	169 ± 20 d	197 ± 23 c	NS
IGFBP-6	120 ± 6 bc	117 ± 8 b	NS

Abbreviations: IGFBP, insulin-like growth factor binding protein; SCD, stearoyl-CoA desaturase; NEFL, neurofilament light polypeptide; ANG-2, angiopoietin 2; SCYA2, small inducible cytokine A2 precursor. Means in a column within a gene group that do not have a common letters (b, c, d, e) differ ($P < 0.01$).

[a] Expression level percentages [levels of either IGF-I, NPY1R or LDHC were arbitrarily set at 100] included: secreted factors: ANG-2 and SCYA2 expressed relative to IGF-I = 100; NPY receptors expressed relative to NPY1R = 100; IGFBPs expressed relative to either IGFBP-2 = 100 or IGFBP-1 = 100. Adipose tissue/nerve markers: preproadipsin expressed relative to IGF-1 = 100, SCD and NEFL expressed relative to LDHC = 100. Means ± S.E.M. of 15% (i.e., 15 pigs) of relative normalized fluorescent intensities from each of 15 MSQ and 15 OSQ adipose tissue microarrays since data from 90, 150 and 210-d-old-pig were combined, * MSQ vs. OSQ, Student's "*t*" test.

Table 5
Gene expression in middle (MSQ) subcutaneous adipose in growing pigs that is not influenced by age: real-time RT-PCR crossing points (C_t) and expression ratios (ratios) for the comparison of 90 and 210 d ages

Ages	90–210 d		
Gene	C_t[a]	C_t	Ratios
IGF-1	18.1 ± 0.3	17.9 ± 0.8	1.3**
ANG-2	21.4 ± 0.1	21 ± 0.4	1.3**
VEGF	16.6 ± 0.2	16.9 ± 0.3	−1.3**
CTGF	20.3 ± 0.6	20 ± 0.8	1.22**
FST	18.7 ± 0.6	19.6 ± 0.2	−1.9*
BDNF	23.6 ± 1.2	26.5 ± 1.9	−5.7*
LEPR	21.3 ± 1.7	22.8 ± 0.2	−2.2**
NYP1R	22.3 ± 0.6	21.7 ± 0.4	1.4**
GHR	13.8 ± 0.7	14.3 ± 0.1	−1.1**

*$P > 0.07$, **$P > 0.1$ for 90–210 d comparison, *P*-values determined by pair-wise fixed reallocation randomization test within REST©.

[a] Values are means ± S.D. of 3 pigs at 90 and 210 d. The C_t were normalized by subtracting 18S values before the calculation of the C_t means. C_t values are inversely correlated with gene expression, i.e., lower C_t values indicate greater gene expression. There was no relaxin detected in MSQ.

Table 6
Gene expression in middle (MSQ) and outer (OSQ) subcutaneous adipose in growing pigs that is not influenced by age: real-time RT-PCR crossing points (C_t) and expression ratios (ratios) for the comparison of 90 and 210 d ages

Ages	90 to 210 d		
Gene	C_t[a]	C_t	Ratios
Depot (MSQ)			
CNTF	25 ± .1	24 ± .1	1.9*
IGF-II	14.6 ± .2	14.8 ± .2	−1.1**
IGFBP5	13.1 ± .4	14.8 ± .6	−3.2*
Depot (OSQ)			
CNTF	18.1 ± .8	19.2 ± .9	−1.9**
IGF-II	14.5 ± .1	14.7 ± .1	−1.1**
IGFBP5	13.2 ± .3	14.4 ± .3	−2.4*
NPY	19.4 ± 1.2	20.2 ± .6	−1.6**

**P* values >0.07, ***P* values >0.10 determined by pair-wise fixed reallocation randomization test within REST© (Relative Expression Software Tool).

[a] Values are means ± S.D. of 3 pigs at 90 and 210 d. The C_t were normalized by subtracting 18S values before the calculation of the C_t means. The C_t values are inversely correlated with gene expression (i.e., lower C_t indicate greater gene expression).

enced by age (Tables 5 and 6). Crossing point values are inversely related to gene expression. Expression of IGF-II, CTGF, CNTF and IGFBP-5 was detected in both OSQ and MSQ depots (Tables 5 and 6) and expression of ANG-2, VEGF, FST, GHR, BDNF, and NYP1R was detected in the MSQ depot (Table 5).

3.4. Age-dependent gene expression in middle subcutaneous adipose tissue and outer subcutaneous adipose tissue in growing pigs

Expression ratios and associated $\Delta\Delta C_t$'s revealed age-dependent decreases in NPY and NPY2R expression and an increase in IGFBP-7 expression in the MSQ depot between 90 and 210 d (Table 7). These parameters also showed that CTGF expression increased in the OSQ depot between 90 and 210 d and 150 and 210 d (Table 7). Real-time RT-PCR crossing point means are also shown (C_t's) for each age and gene (Table 7).

Table 7
Gene expression influenced by age in middle (MSQ) and outer (OSQ) adipose tissue in growing pigs: real-time RT-PCR crossing points (C_t), expression ratios, and associated $\Delta\Delta C_t$'s for the comparison of 90 and 210 d, 150 and 210 d ages

Depot	MSQ		MSQ		MSQ		OSQ		OSQ	
Gene	NPY		NPY2R		IGFBP-7		CTGF		CTGF	
Ages (d)	90	210	90	210	90	210	90	210	150	210
C_t's	22 ± .8	24 ± .4	23 ± .9	25 ± .3	15.4 ± .2	15 ± .1	16 ± .9	13.6 ± .4	15 ± .5	13.6 ± .4
Expression ratios[a]	−3.0		−3.0		1.2		4.5		2.3	
$\Delta\Delta C_t$[a]	1.8		2.02		−0.33		−2.4		−1.38	
P-Values[b]	0.001		0.001		0.03		0.001		0.02	

[a] Expression ratios and associated $\Delta\Delta C_t$'s for MSQ and OSQ adipose tissue samples collected from either day 90 ($n = 3$) and 210 d ($n = 3$) or 150 ($n = 3$) and 210 d ($n = 3$) old pigs, normalized to the endogenous control 18S & calculated by the software tool REST© (Relative Expression Software Tool). Real-time RT-PCR crossing point means are also shown (C_t's) for each age and gene; C_t were normalized by subtracting 18S values before calculation of the C_t means ± S.D.

[b] *P*-Values determined by pair-wise fixed reallocation randomization test within REST©.

4. Discussion

The present study reports, for the first time, expression of 11 genes by subcutaneous adipose tissue from growing pigs utilizing either a combination of gene microarrays and RT-PCR assays or RT-PCR assays alone. Furthermore, ANG-2, VEGF, CNTF, BDNF, IGFBP-5, and FST proteins were identified in media conditioned by neonatal pig adipocyte cultures [13] and were detected in the present study at the gene level in adipose tissue of growing pigs. These collective results (present study; 13) indicate that a number of factors are expressed and possibly secreted by pig adipose tissue throughout growth. The few relevant studies include studies of ANGPTL3 and ANGPTL4 gene expression by porcine adipose tissue [20]. The present study is the only report of NPY1R, NPY2R, CTGF, IGFBP-5, IGFBP-7, FST, CNTF, BDNF, ANG-2 and VEGF gene expression by pig adipose tissue. Furthermore, this study is the only report of IGFBP-7, IGFBP-5, CNTF, CTGF and BDNF gene expression by adipose tissue regardless of species (reviews, 1,3,4) and is one of several reports of NPY gene expression by adipose tissue [19,21].

A combination of gene microarray and RT-PCR analysis or RT-PCR alone demonstrated that gene expression of IGF-I, -II, IGFBPs, IGF and GH receptors did not appreciably change with age in OSQ and MSQ depots from growing pigs. There are no comparable integrated or comprehensive studies of the influence of age on adipose tissue gene expression of these IGF system components in any species. Nevertheless, several relevant studies included a study of isolated porcine adipocytes which demonstrated an age-associated but small increase in IGF-II gene expression [22]. Since we studied adipose tissue per se, changes in non-adipocyte IGF-II gene expression in our study may have precluded detection of a small increase in adipocyte IGF-II gene expression. Regardless, IGF-II/IGF-I gene expression ratios for neonatal porcine adipose tissue [13] and MSQ and OSQ from growing pigs (present study) indicate a relative predominance of IGF-II over IGF-I gene expression throughout adipose tissue development. Predominance of IGF-II gene expression may indicate that adipose tissue may be a greater source of circulating IGF-II than IGF-I. This suggestion is supported by an association of circulating IGF-II levels with adiposity or obesity in the pig [23,24]. A mutation in the IGF-II gene was associated with decreased subcutaneous adipose tissue accretion in pigs [25]. In other studies serum IGF-II levels alone were not associated with back fat accretion in pigs as were the sum of serum IGF-I and IGF-II levels [26]. Regardless, the secretion of IGF-II by adipose tissue would have to be substantiated in further studies. In contrast to our results, several studies have demonstrated age-associated changes in IGF-I [27,28] and IGF-IR [27] gene expression in porcine adipose tissue. Age-associated IGF-I and IGF-IR gene expression may be breed or phenotype-dependent since these studies examined Erhualian (obese), Large White pigs [27] and Yucatan (obese) pigs [28]. Pigs were studied from birth to 180 d of age and the kinetics of the age related changes in these studies were not readily available [27]. Consistent with other reports, significant age-associated increases in leptin and adiponectin gene expression were detected in adipose tissue from these pigs [12].

The present microarray and RT-PCR studies of IGFBP gene expression in adipose tissue from growing pigs are the first meat animal adipose tissue IGFBP gene studies. The expression of IGFBP genes was demonstrated in neonatal porcine adipose tissue with

microarray analysis [13]. A distinct pattern of IGFBP-1, -2, -3, -5 and -6 gene expression was clearly evident for neonatal adipose tissue (Hausman, GJ, unpublished observations; 13) and for MSQ and OSQ depots in growing pigs (present study). A significant distinction of IGFBP-5 from other IGFBPs characterizes the pattern of IGFBP gene expression for adipose tissue from neonatal and growing pigs. Insulin-like growth factor binding protein-5 [13] and IGFBP-1, -2, and -3 proteins [5] have been detected in media conditioned by neonatal adipocytes. Although expression of IGFBP-1, -2, -3, -5 and -6 was not influenced by age or depot, porcine adipose tissue expresses and may secrete IGFBP-5 and other IGFBPs throughout development.

An increase in IGFBP-7 gene expression with age in MSQ was detected despite a small number of replicates. Often identified as IGFBP-rP1/mac 25, IGFBP-7 can bind IGF-I, IGF-II and insulin with a low affinity and IGFBP-7 gene expression was associated with TGF-β and IGF-I stimulated myogenesis [29]. Cortisol increased IGFBP-7 gene expression in cultured osteoblasts [30] whereas cortisol typically decreases IGF and IGFBP expression in osteoblasts [30], adipocytes and preadipocytes [5]. Studies of serum borne IGFBP-7 demonstrated that IGFBP-7 levels were independently associated with IGFBP-1 levels [31]. Insulin-like growth factor binding protein-1 gene expression is also up regulated by glucocorticoids (review, 32) and serum borne and adipose tissue IGFBP-1 levels were up regulated during fetal development [6]. Therefore, glucocorticoid stimulated IGFBP-7 levels may also be associated with some aspect of adipogenesis. The role of IGFBP-7 within the classic IGF axis is not known.

The present study demonstrated that NPY and NPY2R gene expression significantly decreased in MSQ with age in growing pigs. It was also shown that NPY1R gene expression did not change in MSQ with age. These are the only studies of the influence of age on either NPYR or NPY gene expression in adipose tissue. Two relevant studies of domestic animals include studies of cold stress on bovine adipose tissue NPYR gene expression [33] and studies of the influence of NPY treatment on ovine adipose tissue NPY-YI R gene expression [34,35]. In mouse studies, RT-PCR demonstrated that, as in the present study, the NPY (and NPY2R) gene was expressed by adipose tissue [21]. Furthermore, NPY and NPY2R gene expression in adipose tissue responded similarly to diet and cold stress [21]. Immunocytochemical studies in the mouse and rat demonstrated that NPY and NPY2R proteins were present in adipose tissue sympathetic nerves [17,18,21]. Local levels of NPY in adipose tissue could reflect release of NPY from sympathetic nerves [21]. In addition to sympathetic nerves, blood vessel endothelium may be a source of NPY in adipose tissue since endothelial cells secrete NPY and express NPYR and NPY genes [36]. The decrease in NPY and NPY2R gene expression in MSQ with age may reflect a relatively reduced number of nerves and blood vessels attributable to the adipocyte hypertrophy driven expansion in tissue volume during this time.

The expression of genes that encode neurotrophic proteins, i.e., CNTF and BDNF, in adipose tissue from growing pigs was also demonstrated in the present study. For instance, CNTF gene expression was demonstrated in OSQ and MSQ depots and BDNF expression was demonstrated in MSQ. Recent studies indicate the potential significance of the presence of neurotrophic factors in adipose tissue. Neurotrophic factors can modulate adipocyte endocrine function since direct peripheral effects of CNTF include inhibition of adipocyte leptin gene expression and secretion [36]. Furthermore, CNTF directly affects expression of other adipocyte genes and preadipocytes express several CNTF receptor complex components [37,38]. Peripheral NPY treatment directly influences leptin gene expression in ovine adipose tissue [33,34] and NPY was responsible for cross-talk between adipocytes and sympathetic neurons in vitro [39]. It is important to note that that both CNTF and BDNF proteins were secreted by neonatal pig adipocytes [13]. Our collective results (present study; 13) indicate that several neural related proteins that could influence adipocyte leptin expression and secretion are expressed and possibly secreted by pig adipose tissue throughout growth. Furthermore, the greater expression of NEFL in MSQ versus OSQ indicates a greater presence of nerves in the more metabolically active MSQ depot.

This is the first truly developmental study of adipose tissue expression of CTGF and the angiogenic factors, ANG-2 and VEGF. CTGF is a secreted protein known as a fibroblast mitogen but is also an angiogenic factor since it is an endothelial cell mitogen and functions in many other stages of angiogenesis [40,41]. RT-PCR analysis showed that CTGF, ANG-2 and VEGF were expressed by porcine adipose tissue throughout development. Vascular endothelial growth factor and ANG-2 are expressed in adipose tissue from other species and play key roles in adipose tissue angiogenesis (review, 42). With RT-PCR and microarray analysis we demonstrated an age-dependent increase in CTGF gene expression in OSQ with no change in the MSQ depot. The marked age-related increase in CTGF expression in OSQ may indicate either a greater age-associated vascular development or greater connective tissue accretion rate than

in the MSQ. However, the latter possibility is less likely since an expected associated increase in ANG-2 and VEGF gene expression with age in OSQ was not evident. The relative expression of ANG-2 was greater in MSQ than in OSQ but this remains to be validated by RT-PCR analysis. Future studies of these adipose tissue factors should include measures at the protein level to address post-translational control of bioactivity.

The present study is the only report of FST gene expression in adipose tissue despite evidence of FST gene expression in many extragonadal tissues in the rat suggesting a more global role for FST [43]. For instance, FST is expressed by bovine endothelial cells and influences both bovine angiogenesis (review, 44) and bovine preadipocyte differentiation [45]. Further discussion is limited since there is little other relevant information and FST expression in MSQ and OSQ was not influenced by age or depot.

Adipose tissue cells other than adipocytes may be responsible for a large portion of the factors secreted from adipose tissue as discussed previously [12,13]. Approximately 90% of cytokine or adipokine release from adipose tissue could be attributable to non-adipocytes which contrasts to little non-adipocyte release of leptin [46,47]. Further research is necessary to determine the role or involvement of non-adipocytes as sources of porcine adipose tissue secreted factors (review, 1,12,13).

Significant correlations and linear regressions were detected between microarray and matching RT-PCR data which serve to validate the microarray data in this study [12]. A number of diverse genes were used and significant correlations and linear regressions were detected between microarray and matching RT-PCR data in the large diverse group and in smaller functional gene groups [12]. However, age related changes in NPY2R and IGFBP-7 detected with RT-PCR assays were not detected by microarray analysis despite the significant correlations between microarray and RT-PCR data [12].

Our studies demonstrate age related changes in expression of several genes in pig adipose tissue that encode local secreted factors which could impact adipose tissue development per se and leptin expression and secretion. These studies also demonstrate, for the first time, expression of several genes in pig adipose tissue encoding local and endocrine factors which could impact adipose tissue and growth and development in general. Further research is necessary to examine adipose tissue secretion at the protein level throughout growth and development. Regardless, these studies greatly expand the list of pig adipose tissue secreted factors.

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